

RESEARCH ARTICLE

Anti-inflammatory Effects of Topically Applied Azilsartan in a Mouse Model of Imiquimod-induced Psoriasis

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ABSTRACT

Background: Many studies showed a possible exacerbation of psoriasis after exposure to angiotensin receptor antagonists. Azilsartan is a competitive angiotensin II receptor antagonist and has anti-inflammatory effects in various inflammatory disorders.

Objective: Investigate dose-dependent effects of topical Azilsartan on Imiquimod-induced psoriasis in mice.

Methods: Forty-eight mice are allocated into six groups (8 mice per group). They all received Imiquimod for the induction of psoriasis (except Group I, a negative control group). Group II (Induction group) received petroleum gel for six days after induction with Imiquimod. The other groups (III, IV, V, and VI) were given Clobetasol propionate 0.05, 1% Azilsartan, 3% Azilsartan, and a combination of 3% Azilsartan and 0.05% Clobetasol propionate ointments, respectively once daily for six days after induction.

Results: Azilsartan decreased psoriasis area severity index (PASI) score and attenuated the histological manifestations after induction. It significantly decreased the serum and tissue levels of the inflammatory biomarkers (TNF- α , IL-17, IL-23, and NF- κ B), especially when used as an add-on therapy to Clobetasol.

Conclusion: Topically-applied Azilsartan shows anti-psoriatic effects in Imiquimod-induced psoriasis in mice via anti-inflammatory and anti-proliferative activities.

Keywords: Azilsartan, Clobetasol, Imiquimod-induced psoriasis, Inflammation, Mice.

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INTRODUCTION

Psoriasis is a common chronic disease that is genetically transmitted, most likely in a dominant mode, and is characterized by variable penetrations, disfiguring skin disease, and recurring inflammatory and proliferative skin diseases.^{1,2} Psoriasis has a complicated etiology. In psoriatic lesions, the inflammatory infiltrate in psoriatic lesions is largely composed of activated T cells. Based on clinical appearance, the five kinds of psoriasis are plaque, guttate, pustular, inverse, and erythrodermic psoriasis. The chronic kind of plaque is the most common, accounting for 85%–90% of all cases. In psoriasis, an imbalance between the innate and acquired (adaptive) immune systems in the layers of the skin might affect keratinocyte proliferation and differentiation.³ Antigen identification by the innate immune system is aided by a variety of immune cells, including cutaneous dendritic cells, natural

killer [NK] T lymphocytes, and neutrophils. The production of pro-inflammatory cytokines by myeloid dendritic cell monocytes aids the growth and proliferation of T lymphocyte CD4⁺ cells into the Th17 and Th1 cell subsets. In order to maintain the psoriatic inflammation maintenance phase, the adaptive immune system must be activated.⁴ Th17 cells produce cytokines like IL-17, IL-21, and IL-22, which activate the JAK/STAT pathway and encourage the development of keratinocytes in the epidermis. Pro-inflammatory gene transcription is phosphorylated and altered as a result of this activation.⁵ TNF- α , IL-17, and IFN- γ increase keratinocyte proliferation and are necessary in signaling pathways that lead to plaque-type psoriasis development.⁶ NF- κ B is one of the most powerful regulators of pro-inflammatory gene expression. NF- κ B is involved in the production of cytokines such as tumor necrosis factor (TNF- α), interleukin (IL)-1 β , IL6,

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and IL8.⁷ Azilsartan is the most recent angiotensin II receptor blocker (ARB) to be licensed for the treatment of hypertension, and it has a higher potency and fewer side effects.⁸ The effectiveness of Azilsartan in patients with psoriasis has been questioned. However, many clinical and animal investigations have demonstrated Azilsartan's anti-inflammatory properties. In oral mucositis, it successfully lowers IL-1 β , IL-10, and TNF- α levels.⁹ When taken in combination with methotrexate and etanercept, it exhibited clinical improvement in disease activity score and inflammatory biomarkers in patients with rheumatoid arthritis.¹⁰ Azilsartan was found to be effective in treating formalin-induced chronic inflammation and cotton-induced granuloma in animal studies.¹¹ Other studies have found a probable association between psoriasis aggravation and long-term systemic use of ARBs.^{12,13} despite the fact that this effect was not stated in the product's features.

The present study was designed to evaluate the effects of topically applied Azilsartan ointment in two different doses (1% and 3%) alone or as add-on therapy with clobetasol propionate 0.05% ointment on imiquimod-induced psoriasis in mice.

MATERIALS AND METHODS

Drugs and Reagents

IMQ cream (5% w/w) was purchased from Meda Pharmaceutical Co., Germany. Azilsartan powder was purchased from Hyperchem, China. Clobetasol Propionate, 0.05% ointment, was purchased from GSK, UK.

Preparation of Azilsartan Ointment

Azilsartan powder was ground in a mortar with an equal volume of glycerin (10% w/w), which acted as a moistening agent. The mixture was mixed with an equal amount of Vaseline to prepare various dilutions (1% w/w or 3% w/w) through a geometric method until the whole volume of Vaseline was completed to get 100 mL of the mixture.¹⁴

Experimental Design and Animal Models

The study was an experimental animal design performed in the department of Pharmacology, College of Medicine, Al-Nahrain University, from December 2020 to December 2021. The study protocol was reviewed and approved by the Institutional Review Board of the College of Medicine at Al-Nahrain University in accordance with the adopted guidelines for experimental animal care. The animal house of Al-Razi Scientific Center, Iraqi Ministry of Industry and Minerals, provided 48 BALB/c Albino mice aged 8 to 11 weeks with an average body weight of 25–40 g. They were kept in polypropylene cages and fed a regular pellet diet with free access to water. They were allowed to acclimate for 7 days before starting the experiments. The mice were divided into six groups, each with eight mice. After shaving the mice's backs, 62.5 mg of Imiquimod (IMQ) was applied topically to the shaved skin of the mice on a daily basis to produce a scaly inflammatory lesion similar to plaque psoriasis. These lesions have a distinct differentiation pattern as well as enhanced epidermal proliferation. The mice in each group were given the

following treatments: Group I (the control group) was kept as a positive control because it received no therapy. Starting on the seventh day after induction, Group II (the induction group) was given Vaseline petrolatum jell topically for six days. Clobetasol ointment (0.05%) was applied topically once daily to Group III (Clobetasol group) starting on day 7 following induction and continued for 6 days (15). Group IV (1% Azilsartan) was treated for 12 days with 1% Azilsartan ointment topically once daily for six days starting on day 7 following induction.¹⁶ Starting on day seven following induction, Group V (Azilsartan 3% group) was treated Azilsartan 3% ointment topically once daily for six days. Starting on day 7 following induction, Group VI (combination of Clobetasole and Azilsartan 3%) was treated topically with a mixture of Clobetasole 0.05% and Azilsartan 3% ointment once daily for 6 days.

Preparation of Samples and Outcome Evaluation

All mice are sedated with diethyl ether at the end of the studies and slaughtered on day 13 after treatment. Blood samples were taken through heart puncture and then allowed to coagulate. The serum was produced by centrifugation at 2000 rpm for 10 minutes, then stored in an Eppendorf tube at -80°C until the time of ELISA testing.¹⁷ Skin samples were excised and kept in buffer phosphate saline (1:10), then harvested and prepared for tissue homogenate using an electric tissue homogenizer (Staruar, England), centrifuged at 5000 rpm for 10 min to obtain the supernatant, which was stored at -80°C until inflammatory biomarker estimation.¹⁸ Other skin tissue samples were also collected, preserved in 10% formalin, and processed for histological analysis.

Laboratory investigation

Clinical scoring of Skin Inflammation Severity

The PASI system is a modified human scoring system based on the PASI that is used to quantify the severity of skin inflammation throughout the application procedure, with the exception that in the mouse model, the afflicted skin area is not included in the overall score. Erythema, thickness, and scaling are all scored on a scale of 0 to 4, with 0 suggesting no signs, 1 indicating faint signals, 2 indicating moderate signs, 3 indicating marked signs, and 4 indicating extremely marked clinical indicators.^{19,20}

Preparation of Skin Tissue for Histopathological Investigation

The skin tissues were washed with water and prepared for histological analysis. The specimens were dehydrated for 2 hours with various concentrations of ethanol (70, 80, 90, 95, and 100%). After dipping the specimen in liquid paraffin at a temperature of 55–60°C, xylol was added. The tissue specimens were embedded in paraffin and cooled to form paraffin blocks. An automated microtome was used to prepare a 5 μ m thick section. Then, the slices were placed on a slide in a water bath. The technique for staining with hematoxylin and eosin required the tissue to be deparaffinized, rehydrated, placed in hematoxylin, and discolored with access stain. Then

Table 1: Histopathological scoring of the severity of inflammation²³

Items		Score
Keratin	Munro abscess	2.0
	Hyperkeratosis	0.5
	Parakeratosis	1.0
Epidermis	Thinning above papillae	0.5
	Lengthening and clubbing of rete ridges	1.5
	Acanthosis	0.5
	Lack of granular layer	1.0
Dermis	Lymphocytic infiltrate	
	Mild	0.5
	Moderate	1.0
	Marked	2.0
	Papillary congestion	0.5

the sample was dipped in eosin stain, followed by dehydration. A few drops of di-N-butyl phthalate (Di-N-BTP) in xylene (Xylene: DPX) were added to the area, covered with a coverslip, and sealed.²¹ Hematoxylin and eosin were used to stain the specimens. Heat was used to dissolve aluminum potassium sulfate in distilled water. After the alcohol had boiled, the hematoxylin solution was dissolved, and the resulting mixture was removed from the heat. The addition of a very little amount of mercuric oxide was done with care and shaking, and the mixture was then placed immediately in cold water.²¹ A 500 mL eosin solution was made by dissolving 2 g of eosin powder in 25 mL of DW and then adding 475 mL of 100 percent alcohol. The cytoplasm, as well as the extracellular matrix, takes on a crimson or pink stain.²² Epidermal thickening, parakeratosis, hyperkeratosis, Munro abscess, acanthosis, lymphocytic infiltrate, and papillary congestion are semi-quantitative scoring methods for the assessment of mouse model histopathology.²³ On a scale of 0 to 10, Baker's scoring was employed to evaluate the pathological abnormalities, as shown in Table 1.²³

Estimation of TNF-α, IL-17, IL-23, and NF-κB Levels

The Sandwich-ELISA practice module is performed on this ELISA kit to evaluate the levels of TNF-α, IL-17, IL-23, and NF-κB levels in both serum and skin tissue homogenate according to the manufacturer's instructions.²⁵⁻²⁸

Statistical Analysis

The data were analyzed using SPSS version 21 for Windows 10. The results were expressed as a mean ± standard error of means (SEM). Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to compare between means. Chi-square test was used to compare between non-categorical parameters at 0.05 and 0.01 probability.²⁹

RESULTS

Evaluation of Clinical Scores

Upon using Imiquimod for 6 days, the morphological changes began to appear after the first 3 days, with a complete psoriatic picture after 6 consecutive days. Signs of erythema, skin thickness, and scaling increased when compared to the control



Figure 1: Photographs taken on day 14 of the experiment

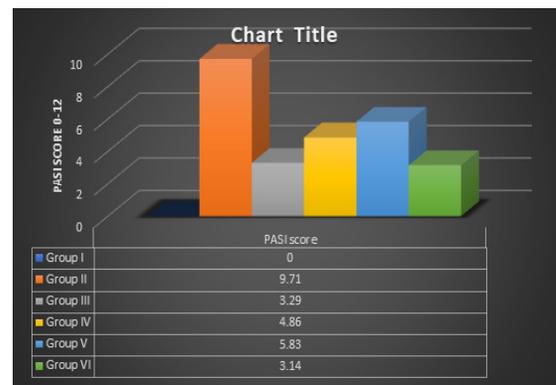


Figure 2: Effect of Azilsartan and Clobetasol on PASI score

group (Group I) as shown in Figure 1, and the PASI score was significantly increased as shown in Figure 2. After this period, tested drugs were used topically, and significant reductions in PASI scores were reported in all other groups.

Histopathological Examination

Skin treated with Imiquimod exhibited pathological changes in the epidermis, including significant hyperkeratosis, acanthosis, Munro abscess appearance, an increase in rete-ridges, and perivascular infiltration of inflammatory cells in the upper dermis, a phenotype characteristic of human psoriatic skin, which were evident in the induction group (group II) and recorded the highest score in Baker scoring system (7.0 ± 0.39). While Clobetasol (Group III), Azilsartan (IV, V) and combined groups (VI) significantly decreased overall Baker scores with 2.0 ± 0.30, 2.86 ± 0.78, 3.92 ± 0.79 and 1.71 ± 0.64, respectively (Table 2). They reduced epidermal thickness by reducing hyperkeratosis and rete-ridges and reducing inflammatory cell infiltration caused by Imiquimod therapy. While the healthy group (I) demonstrated what seemed to be normal stratified epithelium dermis and epidermis thickness, Figure 3 illustrated the histopathological picture for all treated groups.

Estimation of Serum and Tissue Biomarkers Levels Using Enzyme-Linked Immunoassay

Figures 4 and 5 showed the results and statistical analysis of the tissue homogenate and serum levels of IL-17, IL-23, TNF-α

Table 2: Effects of topically applied 1% and 3% Azilsartan ointment on Baker’s score in imiquimod-induced psoriasis in mice compared with 0.5% Clobetasol ointment.

Group No.	Group I (Control)	Group II (Induction)	Group III (Clobetasol)	Group IV (Azilsartan 1%)	Group V (Azilsartan 3%)	Group VI (Azilsartan 3% + Clobetasol)
PASI score	0.0 ± 0.0	7.0 ± 0.39	2.0 ± 0.30**	2.86 ± 0.78**	3.92 ± 0.79**	1.71 ± 0.64**

Values expressed as Mean ± SEM; Induction¹: Imiquimod; ** highly significant between tested group and induction group ($p < 0.01$). p-value was obtained through paired t-test by comparing the means between groups.

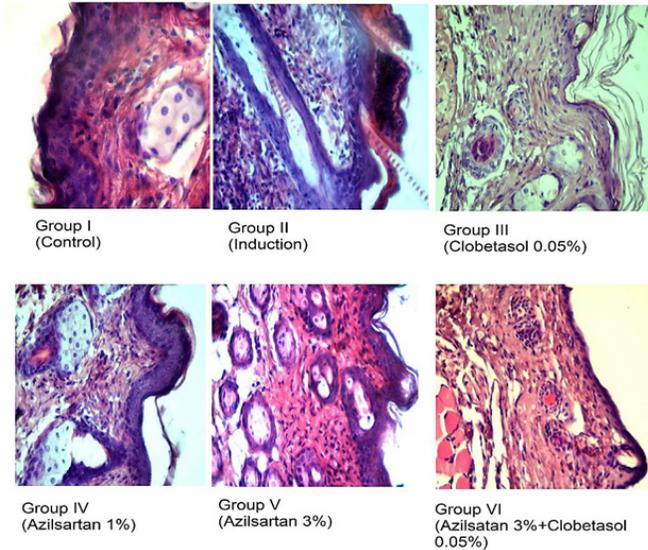


Figure 3: The histopathological picture for all treated groups at 40X magnification, Blue arrow shows keratin layer, Yellow arrow shows epidermal layer, Red arrow indicate dermal layer.

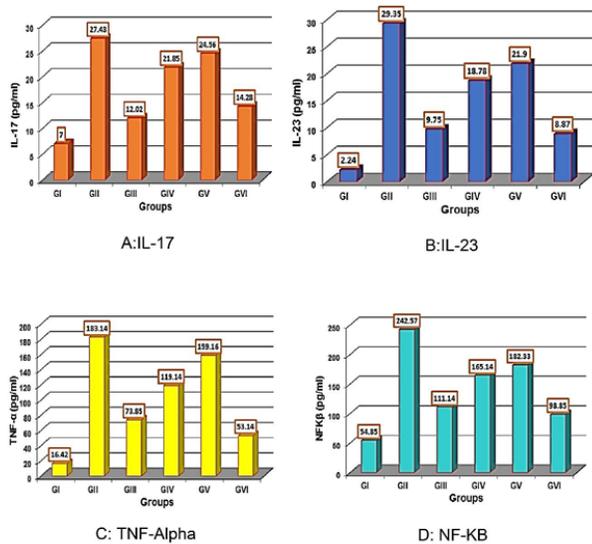


Figure (4): Effects of different dose of Azilsartan on the serum inflammatory biomarkers of Imiquimod-induced psoriasis-like mice. (A) IL-17; (B) IL-23; (C) TNF-α and (D) NF-κB. GI: Control group, GII: induction group, GIII: Clobetasol group, GIV: Azilsartan 1% treated group, GV: Azilsartan 3% treated group and GVI: combination of Azilsartan 3% and Clobetasol 0.05%. Values

and NF-κB, respectively. When comparing the induction group with the control group, the levels of IL-17, IL-23, TNF-α and NF-κB were dramatically raised. The clobetasol-treated group showed significantly lower serum and tissue inflammatory biomarkers levels. Meanwhile, topical treatment with Azilsartan ointment led to different changes in the serum and tissue levels of the inflammatory biomarkers.

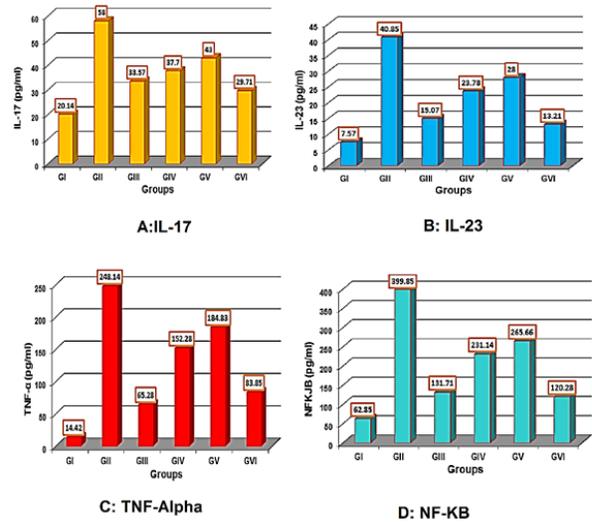


Figure (5): Effects of different dose of Azilsartan on the tissue inflammatory biomarkers of Imiquimod-induced psoriasis-like mice. (A) IL-17; (B) IL-23; (C) TNF-α and (D) NF-κB. GI: Control group, GII: induction group, GIII: Clobetasol group, GIV: Azilsartan 1% treated group, GV: Azilsartan 3% treated group and GVI: combination of Azilsartan 3% and Clobetasol 0.05%. Values are mean ±SEM

There were substantial reductions in serum IL-23, TNF-α, and NF-κB levels in group IV treated with 1% Azilsartan ointment, but no significant reductions in serum IL-17 levels. In contrast, all inflammatory biomarkers were shown to be much lower in the tissue homogenate. Only the serum levels of NF-κB were considerably reduced by the high dose of Azilsartan, whereas the levels of IL-17, IL-23, and TNF-α were unaffected. Except for IL-17, all of the biomarkers examined in the tissue homogenate exhibited a significant drop in their levels. Compared to the other groups, the combination group (group VI) had the best effect on lowering the assessed inflammatory biomarkers.

DISCUSSION

Psoriasis is an autoimmune disease in which the skin’s activated dendritic cells interact with the surrounding keratinocytes on the skin surface, causing redness, scaling, and thickness.³⁰ Due to the simplicity of producing psoriatic skin in these animals with the usual phenotypic of psoriasis, the psoriasis model in mice is an ideal model for inducing psoriasis. The pathophysiological aspects seen in humans, such as cutaneous inflammation, hyperkeratosis, cutaneous infiltration of dendritic cells, CD4 cells, and the anti-oxidant system, are also seen in the mice model.²³ Imiquimod is commonly used in mice to provide a characteristic appearance of psoriasis. However, after six days of treatment, these symptoms go away on their own. These results show that mice

are not genetically harmed and can reverse the inflammatory process in response to Imiquimod treatment.³¹ As a result, we gave IMQ to mice with shaved dorsal skin for 12 days. In the current study, imiquimod use resulted in significant and greater histological changes in group II compared to group I, including hyperkeratosis, parakeratosis, acanthosis, the appearance of Munro microabscess in the epidermal layer, thinning above papillae, lengthening and clubbing over rete ridges, the loss of the granular layer in the majority of samples, and increased inflammatory cell in The PASI score in the Imiquimod-induced group II is significantly higher than in the control group. Because it may act directly on mast cells via IgE-linked pathways, Imiquimod caused erythema. Another theory is that Imiquimod activates mast cells in ways that are not IgE-dependent.³² Imiquimod was shown to significantly enhance all evaluated pro-inflammatory biomarkers and inflammatory signals, resulting in a typical picture of plaque psoriasis, according to ELISA results for serum and tissue inflammatory biomarkers. Many publications have already reported these findings, which demonstrated the influence of Imiquimod on serum and tissue biomarkers.^{19,33-35} Highly potent topical steroids were once thought to be the gold standard for psoriasis treatment, and their efficacy was attributed to many anti-inflammatory and immune-suppressive mechanisms.³⁶ They work in two ways: genomic and non-genomic. The glucocorticoid receptors on the plasma membrane are referred as the genomic target.³⁷ The non-genomic pathway is responsible for glucocorticoids' rapid action. In general, glucocorticoids promote anti-inflammatory gene transcription while decreasing pro-inflammatory gene transcription.³⁸ In the current study, Clobetasol exerted a significant inhibitory effect on the inflammatory response generated by Imiquimod. The optimal reduction in PASI score is evident by a decreasing erythema through vasoconstrictor action of Clobetasol, which is accomplished by blocking the action of vasodilators such as histamine and bradykinin, and a decrease in thickness of epidermal layer by anti-inflammatory and anti-proliferative actions of it.³⁹ The reduction in clinical severity score is accompanied by an improvement in histological changes associated with inflammatory characteristics, as found in the Clobetasol group (Group III) which appeared through reduction in hyperkeratosis, parakeratosis, Munro abscess disappearance, rete ridges, and ameliorating lymphocytic infiltrate. In addition, when compared to induction group II, Clobetasol significantly reduced the inflammatory cytokines IL-17, IL-23, TNF- α , and NF- κ B. Despite the fact that topical Clobetasol is an effective psoriasis treatment, it has been linked to well-known side effects such as striae, atrophy, and telangiectases, as well as systemic side effects.⁴⁰ Because of this limitation, steroidal medications are unsuitable for long-term use, and the demand for novel medicine with fewer side effects is growing. Azilsartan is one of the most recent angiotensin II receptor blockers, and it is typically used as a prodrug (Azilsartan Medoxomil) to treat hypertension and prevent stroke, heart attack, and kidney problems, with varying

effects on skin tissue due to its blocking action on ATII type I receptors in the dermal layers.⁴¹ In the present study, both doses of Azilsartan resulted in a substantial reduction in clinical severity score by reducing erythema, scaling, and back skin thickness, with the low dose showing a greater response. These findings could be related to angiotensin blockers' vasoconstrictor action on blood vessels, which results in less erythema. As previously reported by Huskic et al., it also blocks AT1 receptors in the epidermal layer, resulting in enhanced scaling and thickness of the mouse dorsal skin.⁴² These findings were also supported by the capacity of Azilsartan to ameliorate effect of Imiquimod on histological features and Baker score, as evidenced by decreased hyperkeratosis, Munro abscess, lymphocytic infiltration, and papillary congestion, with no signs of acanthosis or parakeratosis that occurred during the imiquimod induction period. Azilsartan's capacity to block AT1 receptors, which reduces keratinocyte hyperproliferation and differentiation, was linked to these findings.⁴² When it came to the effects of Azilsartan on the inflammatory biomarkers, the 1% ointment exhibited a considerably superior decrease in all the evaluated tissue biomarkers, whereas blood levels of IL-17 did not. In contrast, 3% Azilsartan ointment dramatically reduces serum NF- κ B and tissue IL-23, TNF- α , and NF- κ B levels, but has no effect on IL-17 levels in blood or tissue. The increased efficacy of 1% Azilsartan ointment in lowering inflammatory burden when compared to 3% strength is still inconclusive. Bradykinin levels may rise as a result of ARBs' propensity to operate as ACE inhibitors, which could reverse their anti-inflammatory effects and aggravate psoriasis, according to some researchers.^{43,44} Other researchers believe that ARBs can raise serum angiotensin II levels by blocking angiotensin II's retroactive influence over renin release, and angiotensin II has been shown to enhance keratinocyte proliferation through a receptor that is not affected by antagonists.⁴⁵ To prevent the side effects of high doses of steroids and to get better outcomes through synergism, numerous topical drugs for psoriasis treatment have been widely utilized by combining steroidal and non-steroidal treatments.⁴⁶ In this study, the combination group (group VI) had the best clinical severity score improvement as well as the greatest ameliorating effect on histological features and the Baker scoring system. Furthermore, when administered topically for six days in Imiquimod-induced psoriasis, the combination of Azilsartan 3% and 0.05% Clobetasol exhibited a stronger inhibitory effect on serum and tissue inflammatory indicators than either agent used alone. These results may be linked to Clobetasol and Azilsartan's synergistic anti-inflammatory properties, which will aid in limiting the spread of psoriasis and easing its symptoms.

CONCLUSION

Topically applied Azilsartan, especially at low doses, exhibited anti-psoriasis advantages in imiquimod-induced psoriasis in mice via anti-inflammatory and anti-proliferative actions, according to the presented findings. However, more research

is needed to determine the ideal dose to get the maximal inhibitory impact while minimizing the risk of a psoriasis flare-up.

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CONFLICT OF INTERESTS

No conflict of interests declared by the

AUTHORS FUNDING SOURCE

No specific fund provided Data

SHARING STATEMENT

The corresponding author can supply the data source in response to a specific request.

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